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Transforming-growth-factor-β activation elements in the distal promoter regions of the rat α1 type I collagen gene

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We have located a *cis*-acting element (α1-TAE) within the promoter sequences of the rat collagen α1(I) gene (COL1A1) 1600 bases upstream of the transcription start site which mediates transcriptional activation by transforming growth factor β (TGF-β). The functional significance of this region was established by (1) deletion analysis of the α1(I) promoter cloned upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene and (2) by co-transfection of promoter constructs with double-stranded oligonucleotides. DNA-mobility-shift assays with radiolabelled α1-TAE demonstrated increased nuclear binding activity after TGF-β stimulation. Oligonucleotides encoding the α1-TAE, additional upstream regions within the α1(I) promoter, as well as consensus nuclear-factor-1 (NF-1) sequences, competed with the α1-TAE sequence. The two collagen type I genes are stimulated by TGF-β through different regions of their promoters.

INTRODUCTION

Collagen type I, composed of two protein chains, α1(I) and α2(I), is expressed by several cell types and represents the most abundant member of the collagen family [1]. After injury to tissues, type I collagen gene expression by fibroblasts is stimulated producing scar tissue or fibrosis. Transforming growth factor β (TGF-β) is a multifunctional growth factor that stimulates collagen type I production [2–6] and is implicated as an active factor in the wound-healing response [7–9].

We have demonstrated previously that human embryonic-lung fibroblasts stimulated by TGF-β produce increased type I collagen protein without stimulation of cell proliferation [4]. There is a concentration-dependent increase in the expression of α1(I) mRNA [5] resulting from an increase in transcription and an increase in mRNA stability [8]. Transcription as measured by 'nuclear run-off' studies demonstrate a 2-fold increase in the production of α1(I) gene transcripts [6].

Transcriptional stimulation occurs through complicated interactions of *cis*-acting DNA sequences and *trans*-acting protein factors. Several regulatory regions have been identified in the collagen type I genes, both within the promoter [10–13] and in the first intron [14–16]. In particular, a TGF-β activation region (α2-TAE) [TCG(N)$_5$GCCAAG] has been located in mouse α2(I) gene approx. 300 bases before the transcription start site [9]. This TGF-β activating element (α2-TAE) sequence on the template strand is similar to, but not exactly the same as, a consensus sequence for nuclear-factor-1 (NF-1)-binding proteins [17]. NF-1 consensus sequences compete for binding at this site. In addition, histone H1 binds to this sequence [18].

The previously described full sequence of the TAE in the α2 promoter (α2-TAE) is not located in the analogous region of rat α1(I) promoter sequence [10]. In order to identify the TAE sequences within the α1(I) promoter region, we have constructed chimaeric genes containing several regions of the promoter with 115 bases of the first exon fused to the chloramphenicol acetyltransferase (CAT) gene. These sequences were transfected into human lung fibroblast cells in order to determine which regions are responsive to TGF-β. In the present paper we describe a responsive α1-TAE region located upstream at −1627 in the α1(I) collagen promoter.

MATERIALS AND METHODS

Plasmid construction

Several α1(I) promoter constructs cloned upstream of CAT were used in transient transfection assays. The largest promoter construct was the previously described construct, ColCat 3.6 [10], which contains 3520 bp of rat α1(I) collagen promoter followed by 115 bp of the rat α1(I) first exon cloned upstream of CAT gene within the pUC 13 vector. Smaller constructs were made by deletion of fragments from ColCat 3.6 plasmid, as indicated in Fig. 1. ColCat 2.4 was constructed by digestion of ColCat 3.6 with the restriction endonuclease *Hin*dIII to delete a 1224 bp fragment between −3520 and −2296. ColCat 1.7 was constructed by *Pst*I digestion of ColCat 3.6 to delete 1848 bp of rat promoter from −3520 to −1672. ColCat 0.9, constructed from a *Pvu*II digestion of ColCat 3.6, contains a 2576 bp deletion of the rat promoter from −3520 to −944. ColCat 0.4 was constructed by the double digestion of ColCat 0.9 with the restriction endonucleases *Pst*I and *Mst*II (*Sau*I), deleting 494 bp from −944 to −450. All the above constructs were re-ligated with T4 DNA ligase. The ColCat 0.4 plasmid was blunt-ended with T4 DNA polymerase enzyme before re-ligation.

Finally, ColCat 0.2 was constructed from a *Bgl*II digestion of ColCat 0.9 to produce a minimal promoter containing 225 bp of the promoter along with the 115 bp of the first exon. The *Bgl*II fragment was first cloned into pBluescript (Stratagene Cloning Systems, La Jolla, CA, U.S.A.). The restriction-enzyme sites for *Hin*dIII and *Xba*I in the multicloning region were used to reclone the promoter fragment into a *Hin*dIII/*Xba*I-digested CAT vector.

Cell cultures

IMR-90 cells (human embryonic lung fibroblasts; Institute for Medical Research, Camden, NJ, U.S.A.) were maintained in Dulbecco's modified Eagle's medium with 370 mg of $NaHCO_3$/

Abbreviations used: TGF-β, transforming growth factor β; CAT, chloramphenicol acetyltransferase; NF, nuclear factor.

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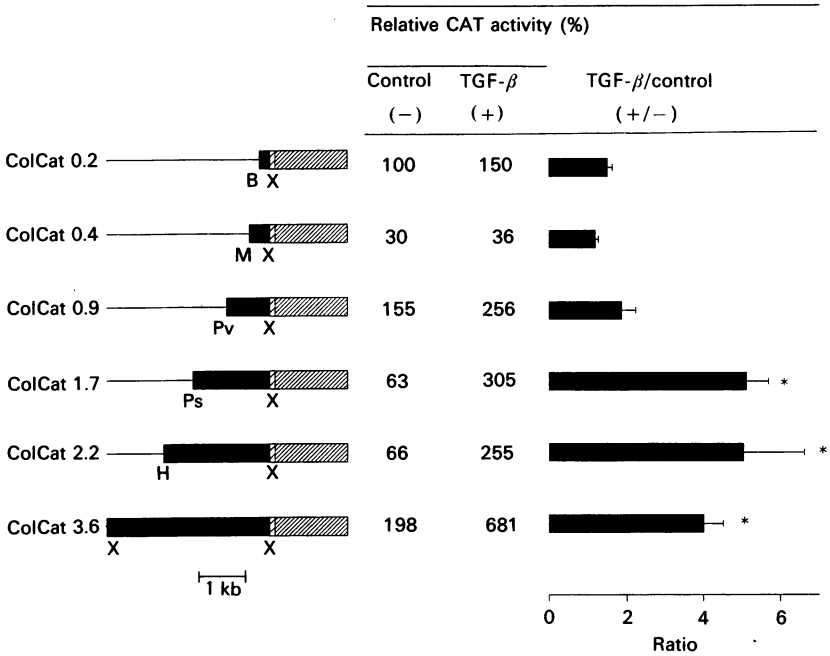

Fig. 1. Diagram of constructs and summary of the results of CAT expression with six α1(I) collagen promoter constructs with (+) and without (−) TGF-β stimulation

Constructs used in these experiments are drawn to scale (left graph). Black bars represent the α1(I) promoter in the construct, widely hatched bars represents the 115 bases of first exon, closely hatched bars represent the CAT gene with SV40 polyadenylation site, and the thin lines represent the deleted promoter fragments. Restriction sites are indicated as follows: X is *Xba*I; B is *Bgl*II; M is *Mst*II; Pv is *Pvu*II; Ps is *Pst*I, H is *Hin*dIII. A 1 kb scale bar is shown below. Constructs were transiently transfected into human lung fibroblasts and CAT activity was assayed as described in the Materials and methods section. Acetylated and non-acetylated chloramphenicol, separated by t.l.c., were revealed by X-ray autoradiography and the radioactive areas of the thin-layer chromatogram were removed and counted for radioactivity in scintillation fluid. The percentage acetylation was calculated as (radioactivity in the acetylated areas)/(total radioactivity extracted from the thin-layer plate) × 100. The relative CAT activity is expressed as the percentage acetylation normalized to the expression of the smallest promoter construct ColCat 0.2 without stimulation by TGF-β. The number of CAT assays used for each determination are between four and eight separate experiments. The ratio of the stimulated (+TGF-β) divided by the unstimulated (−TGF-β) acetylation radioactivity is plotted as a bar graph at the right-hand side of the Figure. Error bars represent S.E.M., and *indicates a significant ($P < 0.05$) difference between the ColCat 0.9 and larger constructs determined by using Student's *t* test.

100 ml, 10% (v/v) fetal bovine serum (Hyclone, Logan, UT, U.S.A.), 100 units of penicillin/ml, 10 μg of streptomycin/ml and 1 ml of 100 mM-sodium pyruvate/100 ml in a humidified 5%-CO_2 incubator at 37 °C.

Transient transfections

Confluent fibroblasts were treated with 1 ml of trypsin (0.5 g/l)/EDTA (0.2 g/l) and plated at a density of (8–9) × 10^5 cells/100 mm^2 tissue-culture dish. Each dish contained 10 ml of 10%-(v/v)-serum medium and was incubated in 8% CO_2 at 37 °C. After 24 h the plasmid DNA was transfected into fibroblasts by calcium phosphate precipitation [19]. CAT plasmids (10 μg) were co-transfected with β-galactosidase [20] (pRSV-β-gal) (5 μg) to normalize transfection efficiency. Cells were 15%-(v/v)-glycerol-shocked for 45 s [19] 16 h after addition of plasmids. Cells grown to confluence (6 h) were rendered quiescent by decreasing the serum concentration to 0.4%. Cells were then stimulated with TGF-β (2 ng/ml of media) (Research Diagnostic Systems, Minneapolis, MN, U.S.A.) for 24 h before harvest. The TGF-β (1 μg) was dissolved in 1 ml of 4 mM-HCl with 1 mg of BSA before addition to the medium. This solution was added to controls without TGF-β. Cells were harvested in 1 ml of 0.01 M-EDTA/1.0 M-NaCl/0.1 M-Tris/HCl, pH 8.0.

In certain experiments, oligonucleotides (1–20 μg) were also co-transfected with plasmids to determine if they could compete for factors stimulating promoter activity in the presence of TGF-β. Oligonucleotides were synthesized by Oligo etc. (Guilford, CT, U.S.A.). The double-stranded oligomer found in α1(I) promoter sequence (α1-TAE) beginning at −1627 was constructed by annealing complementary oligonucleotides (5′-AGCTTGCCCACGGCCAAGA-3′ to 5′-AGCTTCTTGGCCGTGGGCA-3′) in the presence of 200 mM NaCl by heating to 95 °C for 7 min and cooling slowly to 4 °C. Both these sequences contain cohesive *Hin*dIII ends for purposes of subcloning into pBluescript. A sequence found in α2(I) first exon, (5′-CTGCAGATTGGCATGTTGCTAGGC-3′) was annealed to its complementary sequence and used as a non-specific control.

Enzyme assays

Cell extracts were centrifuged for 3 min (at 1500 *g*), resuspended in 100 μl of 0.25 M-Tris/HCl, pH 7.8, and sonicated for 15–20 s with a Branson model 450 sonifier. Total protein was measured by Bradford's method with Coomassie Blue G-250 [21].

CAT activity was measured as described by Gorman *et al.* [19]. Radiolabelled chloramphenicol (0.25 μCi of D-*threo*-[*dichloroacetyl*-1-^{14}C]chloramphenicol) and 4 mM-acetyl-CoA were added to samples containing 100 μg of protein or standard enzyme in 150 μl of 0.25 M-Tris/HCl, pH 7.8. The samples were incubated at 37 °C for 4–6 h, which was within the linear range for these samples. After ethyl acetate extraction, the chloramphenicol and the acetylated products were separated by t.l.c. for 1.25 h in chloroform/methanol (19:1, v/v). The thin-layer plates were exposed to X-ray film for autoradiography. The radioactivity in the acetylated and non-acetylated areas was removed from the

thin-layer plates, placed into scintillation counting fluid and measured in a Beckman scintillation counter.

Experimental transfection efficiency was determined by co-transfection with β-galactosidase gene [20]. Sonicated cell extract (30 μl) or 3 units of β-galactosidase enzyme was incubated for 16 h at room temperature in 1.0 mM-$MgCl_2$/15 mM-β-mercaptoethanol/3.0 mM-2-nitrophenyl β-D-galactopyranoside/70 mM-sodium phosphate buffer, pH 7.4. The reaction was terminated with 500 μl of 1 M-Na_2CO_3, and the A_{410} was measured.

DNA mobility-shift assays

Four confluent T150 mm^2 flasks were prepared for nuclear extracts with or without TGF-β (2 ng/ml) (Research and Diagnostic Systems, Minneapolis, MN, U.S.A.) as described for transfection studies. Nuclear protein was extracted (approx. 1×10^8 cells) by the method of Dignam *et al.* [22] with the addition of extra proteinases [the extraction buffer was 0.42 M-NaCl/1.5 mM-$MgCl_2$/0.2 mM-EDTA/0.50 mM-dithiothreitol/25% (v/v) glycerol/0.5 mM-phenylmethanesulphonyl fluoride/leupeptin (50 μg/ml)/1% (w/v) aprotinin/20 mM-Hepes, pH 7.9]. Protein concentration was determined by Bradford's method described above.

Synthesized complementary α1-TAE oligonucleotides used above for co-transfection studies were radiolabelled with T4 polynucleotide kinase. Various amounts of nuclear protein extracts (2–20 μg) were incubated for 30 min at room temperature with radiolabelled double-stranded oligonucleotide [(260–270) $\times 10^3$ c.p.m./0.5 pmol in each assay] in buffer containing 450 mM-KCl, 5 mM-EDTA, 5 mM-dithiothreitol, 25% (v/v) glycerol and poly(dI-dC) (0.5–3 μg). In some assays, non-radiolabelled double-stranded oligonucleotides were present as competitors in 2–250 molar excess. The following regions within the rat α1(I) promoter were used as competitors in gel shift assays: α1-TAE (−1627 to −1648), non-specific region (−1457 to −1464), upstream competitor 1 (−2965 to −2981), upstream competitor 2 (−2372 to −2390) and adenovirus NF-1 site. A mutated TAE oligonucleotide sequence was also synthesized and tested in competition assays. Six bases were altered according to our preliminary methylation-interference assays (results not shown). The actual sequences for all competing oligonucleotides are listed in the legend to Fig. 6 (below). Incubation mixtures were separated on 6%-(w/v)-polyacrylamide gels (20:1 acrylamide/bisacrylamide ratio) in low-ionic-strength buffer [$\frac{1}{4} \times$ TBE; 22.25 mM-Tris/borate/22.25 mM-boric acid/500 μM-EDTA) for 2–3 h at 10 V/cm^2. The gels were fixed in acetic acid/methanol/water (1:1:8, by vol.), dried under vacuum, and exposed to X-ray film for 3 h.

RESULTS

Sequences from the 5′-flanking region of the collagen α1(I) gene were tested for transcriptional activity and responsiveness to TGF-β by transient transfection of human lung fibroblasts. The constructs used are shown in Fig. 1. A typical CAT assay from cells transfected with the longest construct (ColCat 3.6) with and without TGF-β stimulation is shown in Fig. 2. Duplicate assays represent separate transfected dishes of cells. Radioactivity was quantified by scintillation counting of the radioactive areas from the thin-layer chromatogram after autoradiography. The acetylation/μg protein and the percentage acetylation was calculated.

The promoter activity of the ColCat 3.6 plasmid was markedly stimulated (average of 4-fold) by TGF-β. In order to localize the TAE sequence, several 5′ deletions were made in the promoter. The constructs used are shown in Fig. 1 and described in the Materials and methods section. The CAT activity given in Fig. 1 is expressed relative to the smallest construct (ColCat 0.2) which contains 226 b of promoter, including the CCAAT and TATA areas. This region of the promoter has been considered the proximal promoter for the mouse α1(I) gene [12].

The fold induction of CAT activity by TGF-β is expressed as a ratio of TGF-β stimulated over unstimulated values and plotted in Fig. 1. There were four to eight separate experimental

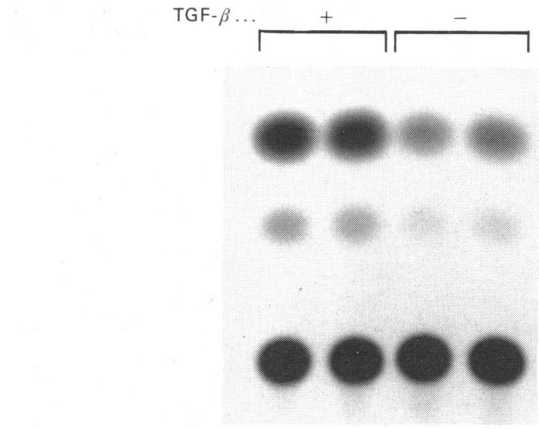

Fig. 2. Representative assay for CAT activity of the α1(I) collagen promoter (plasmid ColCat 3.6) with (+) and without (−) TGF-β stimulation

This is a typical assay using the ColCat 3.6 plasmid (see Fig. 1 and the Materials and methods section for more details). Each duplicate represents a CAT assay with 100 μg of protein in a homogenate from a separate dish of cells. 'Radioactivity (c.p.m./μg)' is the amount of radioactivity extracted from the acetylated areas per μg of protein. The percentage acetylation was calculated as (radioactivity in the acetylated areas)/(total extracted from the thin-layer plate) × 100.

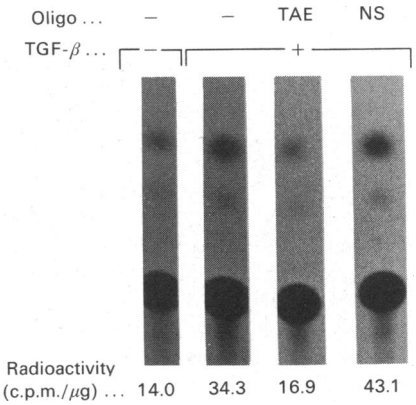

Fig. 3. Oligonucleotide competition *in vivo* abrogates the TGF-β stimulation of ColCat 1.7 plasmid

Human lung fibroblasts were transfected as described in Fig. 1 by using 10 μg of ColCat 1.7 with (+) and without (−) TGF-β stimulation either along '(Oligo−)' or with 10 μg of TAE, rat α1(I) promoter sequence −1627 to −1643 (5′-TTGCCCACGGCCAAG-3′) with *Hin*dIII overhang, or non-specific (NS) oligonucleotide, α2(I) first exon sequence +193 to +209 5′-CTGCAGATT-GGCATGTTGCTAGGC-3′.

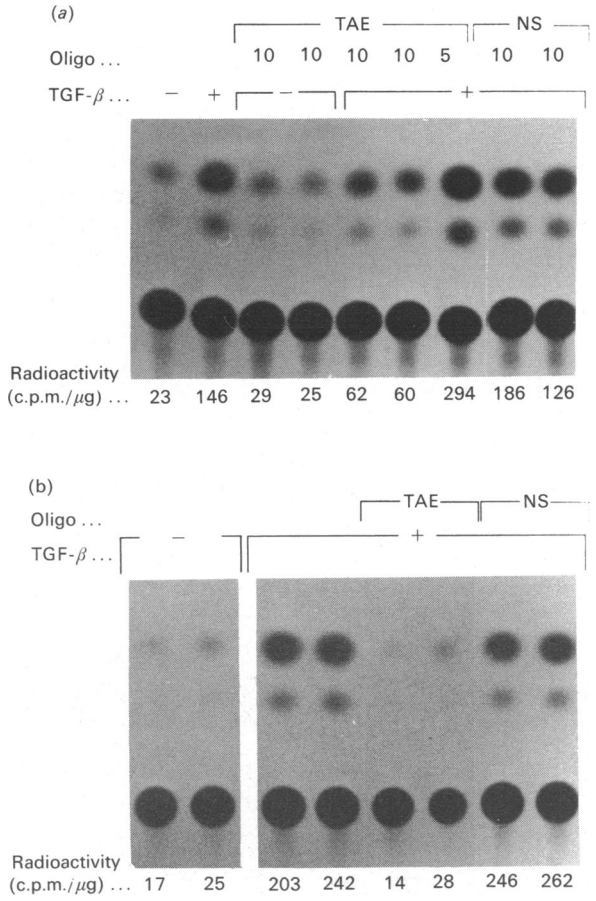

Fig. 4. Oligonucleotide competition *in vivo* requires more oligonucleotide to abrogate TGF-β stimulation of ColCat 3.6 plasmid

(*a*) Human lung fibroblasts with (+) or without (−) TGF-β stimulation were co-transfected as described in Fig. 1 with 10 μg of ColCat 3.6 plasmid and various amounts (5 or 10 μg) of TAE or non-specific (NS) oligonucleotides ('Oligo'). (*b*) In a separate experiment 20 μg of TAE or non-specific (NS) oligonucleotide was co-transfected with ColCat 3.6 plasmid.

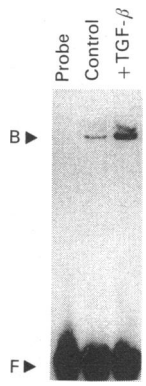

Fig. 5. DNA-mobility-shift assay with TGF-β-stimulated lung fibroblast nuclear extracts demonstrate increased protein binding

Nuclei from human lung fibroblasts (1×10^8 cells) with and without TGF-β stimulation were isolated and proteins were extracted with 0.42 M-NaCl/1.5 mM-$MgCl_2$/0.2 mM-EDTA/0.50 mM-dithiothreitol/25% (v/v) glycerol/0.5 mM-phenylmethanesulphonyl fluoride/leupeptin (50 μg/ml)/1% (w/v) aprotinin/20 mM-Hepes, pH 7.9. Nuclear proteins (2 μg) were incubated for 30 min at room temperature with radiolabelled oligonucleotide (260000 c.p.m./0.5 pmol in each assay) in buffer containing 450 mM-KCl, 5 mM-EDTA, 5 mM-dithiothreitol, 25% (v/v) glycerol and poly(dI-dC) (0.5 μg). Incubation mixtures were separated on 6% (w/v)-polyacrylamide gels in low-ionic-strength buffer (22.25 mM-Tris/borate/22.25 mM-boric acid/500 μM-EDTA) for 2–3 h at 10 V/cm². The gels were fixed in acetic acid/methanol/water (1:1:8, by vol), dried under vacuum, and exposed to X-ray film for 3 h. B, bound DNA–protein complex; F, free DNA.

points averaged for each value shown with S.E.M. bars. TGF-β induced a greater-than-4-fold increase in CAT activity using the 3.6 ColCat, 2.4 ColCat, and 1.7 ColCat constructs. In contrast, constructs containing 0.9 kb or less were minimally stimulated by TGF-β (1–1.7-fold). ColCat 0.4, which contained the region analogous to the mouse α2(I) TGF-β-responsive region, showed no TGF-β stimulation and a decrease in basal CAT activity consistent with the presence of a negative *cis*-acting element [23].

The difference in TGF-β stimulation between the smaller promoters and the larger promoters suggested that a major TGF-β-activating *cis*-acting element was located between 0.9 and 1.7 kb of the α1(I) transcriptional start site. Examination of the rat promoter sequence between −1627 and −1643 revealed a possible α1-TAE region that contained the 3′-portion of the NF-1 canonical sequence (GCCAAG) [17] found in the mouse α2(I) TGF-β-responsive region (α2-TAE).

To test the functional importance of the α1-TAE sequence, complementary oligonucleotides to this region were annealed and co-transfected with plasmids. The α1-TAE sequences in the α1(I) promoter were compared with an unrelated sequence from the α2(I) first exon. Fig. 3 indicates that 10 μg of rat α1-TAE sequence (between −1627 and −1643) inhibited the TGF-β stimulation of ColCat 1.7 construct, whereas an unrelated non-specific sequence did not alter the TGF-β response. The rat oligonucleotide was able to inhibit TGF-β stimulation with as little as 2 μg α1-TAE oligonucleotide, whereas 1 μg was not active (results not shown). This suggests that α1-TAE sequence is the TGF-β-activating element in the region between 0.9 and 1.7 kb.

Dose–response studies indicate that more α1-TAE oligonucleotide molecules were necessary to inhibit TGF-β stimulation of the ColCat 3.6 than the ColCat 1.7 construct, suggesting multiple active sites within the promoter. Fig. 4 shows the inhibition of the TGF-β response using the ColCat 3.6 construct with various concentrations of oligonucleotides. When using ColCat 3.6 construct, 5 μg of α1-TAE did not effect the TGF-β response of ColCat 3.6, whereas it reduced the response of ColCat 1.7. In addition, 10 μg of oligonucleotide α1-TAE inhibited the TGF-β response by 58%, whereas this concentration completely inhibited the TGF-β response of ColCat 1.7 construct. In contrast, 20 μg of α1-TAE completely inhibited the TGF-β response of ColCat 3.6 and the non-specific oligonucleotide had no effect (Fig. 4*b*). The addition of α1-TAE oligonucleotides to cells without TGF-β did not alter basal CAT activity.

In order to examine the binding of α1-TAE sequences to nuclear proteins, the α1-TAE sequence was end-labelled and incubated with equal amounts (2 μg) of nuclear proteins extracted from control and TGF-β stimulated nuclei. DNA-mobility-shift assays demonstrated that TGF-β-stimulated nuclear extracts contained greater-than-3-fold more protein binding activity than unstimulated nuclei samples (Fig. 5). All TGF-β-stimulated nuclear extracts (five experiments) contained more DNA-binding activity than unstimulated nuclear extracts. The electrophoretic patterns obtained in the mobility-shift assays were similar with

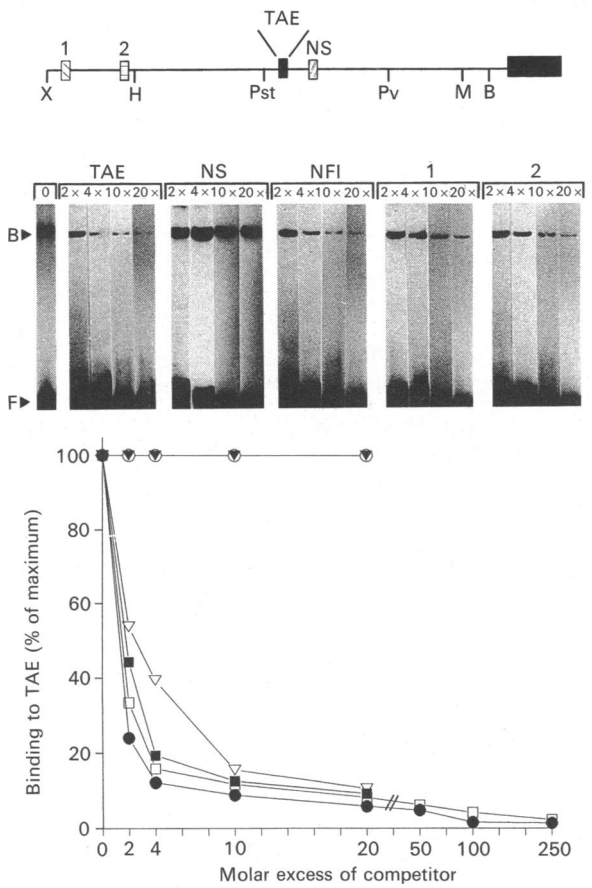

Fig. 6. Oligonucleotide competition for protein binding to α1-TAE in DNA-mobility-shift assays

Conditions in this experiment were similar to those of Fig. 5. Each assay mixture contained 2 μg of nuclear protein extract and 0.5 μg of poly(dI-dC). The specific radioactivity of the radiolabelled α1(I)-TAE was 270000 c.p.m./0.32 pmol in each assay. The oligonucleotide competitors in 2–250-fold molar excess were added to the nuclear extracts, followed by the addition of the radioactive oligonucleotide. An autoradiogram was obtained after 3 h exposure. The shifted oligonucleotide bands were removed, counted, and values are plotted as percentages of maximum binding without inhibitors versus molar excess of inhibitor. Sequences listed below (with additional *Hin*dIII linkers) were used as competitors and their position in the promoter is shown schematically in the top diagram. Symbols for sequences: ●, TAE (5′-TGC CCACG GCCAAGA); ○, mTAE (5′-TGT GCGCG GCCGCT); ■, NS (5′-ACG TTA); △, adenovirus NF-I (5′-TGC GTACCC GCCAAGA); □, competitor 1 (5′-TGG TCTGCA GCCAAG); ▲, competitor 2 (5′-TGG GTACCC GCCAAT). Other symbols: X, *Xba*I; H, *Hin*dIII; Pst, *Pst*I; Pv, *Pvu*II; M, *Mst*II; B, *Bgl*II; in the centre part of the Figure, B is bound DNA-protein complex and F is free DNA.

various protein concentrations (between 2 and 20 μg) or poly(dI-dC) concentrations (between 0.4 and 3 μg).

In order to investigate the specificity of the α1-TAE binding, competition studies were performed. Unlabelled α1-TAE competed with itself for protein binding (TAE; Fig. 6), whereas a sequence further downstream (−1457 to −1464) in the rat promoter did not compete at 2–20-fold molar excess (NS; Fig. 6). The TAE sequence was mutated at potential protein contact points according to methylation interference assays (results not shown). These mutated oligonucleotides did not compete for DNA binding (Fig. 6). Since the α1-TAE is similar to the NF-1 sequence, adenovirus NF-1 sequence was used and competed effectively with the TAE sequence (NFI; Fig. 6).

There are two other sequences containing the 3′ region of the NF-1 site in the rat promoter found upstream in the region between 2.2 kb and 3.6 kb shown schematically in Fig. 6. One or both of these sites may also be interacting with the competing oligonucleotide in transfection-competition studies, since more oligonucleotide was necessary to abrogate the TGF-β response of the ColCat 3.6 plasmid. Therefore these regions were tested as competitors for binding in DNA-mobility-shift assay. The two upstream oligonucleotide sequences (1 and 2; Fig. 6) competed almost as well as the NF-1 sequence. In order to quantify the competition assays, the shifted DNA–protein bands were removed, counted for radioactivity in a scintillation counter, and the percentage radioactivity (of maximum bound without competitor) plotted (Fig. 6). The order of competition affinities were α1-TAE > adenovirus NF-1 > competitor 2 > competitor 1. The mutated α1-TAE and non-specific competitor from another region of the promoter (−1400) demonstrated no competition.

DISCUSSION

Our 5′-deletion analysis of the α1(I) promoter indicates that the major *cis*-acting TGF-β responsive element (α1-TAE) in the rat α1(I) promoter is located approx. 1600 bases upstream of the transcription start site. This is a different position, further upstream, than the α2-TAE found in the mouse α2(I) promoter between −308 and −322 [9]. Most important, a 400 b α1 promoter construct (ColCat 0.4), which contains a sequence corresponding to α2-TAE, was not stimulated with TGF-β, consistent with recent findings demonstrating a proximal inhibiting element in the α1(I) promoter (−339 to −361) [23]. Therefore TGF-β transcriptional activation sequences are not located in the same region for the α(I) and α2(I) genes. The distance between the TGF-β-responsive elements and the start site of transcription could explain subtle differences in transcriptional regulation between these two genes [5].

The previously reported α2-TAE sequence [9] is similar to NF-1 consensus sequence. We tested a site between −1627 and −1643 within the region most responsive to TGF-β, which we have termed α1-TAE. The functional significance of the α1-TAE region was demonstrated by co-transfection of a double-stranded α1-TAE oligonucleotide with ColCat 1.7 or ColCat 3.6 with and without TGF-β stimulation. The α1-TAE-inhibited TGF-β stimulation by both active constructs (ColCat 1.7 and ColCat 3.6). More oligonucleotide was necessary to completely inhibit the TGF-β response with the 3.6 kb promoter, suggesting interactions with sites further upstream. There are other NF-1-like sequences present upstream in the rat α1(I) promoter which could be involved: a consensus NF-1 site (at −2300) and a similar sequence (at −2900) found in our largest construct, ColCat 3.6.

Our DNA-mobility-shift assays suggest that, in co-transfection studies, α1-TAE oligonucleotides are abrogating the TGF-β response by competing with the nuclear DNA-binding proteins. The proteins binding to the α1-TAE sequence are most likely members of the NF-1 family of proteins [24], since adenovirus NF-1 consensus sequence competes for DNA binding proteins with α1-TAE, although other factors could bind to this sequence forming protein complexes [25]. Members of the NF-1 family have been identified as multifunctional proteins (CTF/NF-1) [26] which bind to several sequences with different binding affinities [27]. Other members of this family with considerable sequence similarity to the CTF/NF-1 protein have been isolated as proteins binding to the 5′ region (TGGCA) of the original canonical NF-1 site [28–30]. It is not clear whether the factors binding to our sequence are unique or similar to these cloned sequences or isolated proteins.

Several possibilities exist for the transcriptional response mechanism of TGF-β. Chromatin structure could be altered by TGF-β, since histone H1 protein has been shown to bind to the α2-TAE site [18]. Nucleosome positioning has been shown to inhibit NF-1 accessibility to its binding site [31]. Alternatively, the NF-1 proteins could be bound in inhibitor complexes similar to nuclear factor κB (NF-κB) [32,33]. Further studies are necessary to determine the exact mechanism for activation of DNA binding.

We [34] and others [35] have demonstrated that cycloheximide does not alter collagen mRNA levels during TGF-β stimulation indicating that protein synthesis is not necessary for TGF-β response. The present studies demonstrate that TGF-β alters DNA-binding activity. Increased DNA binding without protein synthesis could be the result of post-translational modifications. TGF-β has been shown to prevent the phosphorylation of retinoblastoma protein, which keeps the protein in an active state [36], and to increase dephosphorylation of proteins [37]. Taken together, the most likely mechanism for increased DNA protein binding to α1-TAE is a post-translational modification of a pre-existing protein.

Several investigators have demonstrated that TGF-β stimulates production of collagen in several cell types [2–6]. The mechanism for this stimulation involves alterations at several levels of gene expression, including increased stability of mRNA [2,3], transcription [2,6], and translation [5]. Each small increase amplifies the amount of collagen synthesized. We have demonstrated only a 2–3-fold increase in transcription by nuclear run-on assays or steady-state levels in human fibroblast cell lines used here [5,6]. Therefore, the 3–4-fold increases seen in these CAT assays is significant.

It is noteworthy that other *cis*-acting elements found in stromelysin and collagenase promoters respond to TGF-β through an inhibitor element involving the *Fos* gene [38,39]. The TGF-β response element in the stromelysin and collagenase promoters functions as a transcriptional silencer. These particular genes code for proteins that degrade collagen. Therefore, increased synthesis of collagen coupled with a decrease in enzymes that degrade collagen will further amplify the accumulation of collagen protein.

In summary, we have shown that a TGF-β activation element exists in the α1(I) promoter. This element is similar to the previously characterized α2(I)-TAE element, but is located in a different region further upstream (approx. 1600 bases) from the transcriptional start site. In addition, there are other sites within the promoter that could be interacting with the same DNA-binding protein. DNA-mobility-shift assays indicate that more nuclear protein is binding to the TAE-element in TGF-β stimulated nuclei extracts than control nuclei extracts. Since cycloheximide does not alter the TGF-β induction of α1(I) mRNA levels, TGF-β probably activates the DNA-binding potential of a pre-existing binding protein without new protein synthesis.

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